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Localization and Quantification of Gonad Serotonin During Gametogenesis of the Surf Clam, *Spisula solidissima*

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Abstract. In the surf clam, *Spisula solidissima*, serotonin was reported to induce spawning when injected into the gonads. At nanomolar concentrations, it facilitates the fertilizability of oocyte by sperm, at micromolar concentration, it triggers the meiotic maturation of prophase 1-arrested oocytes, thus mimicking the effect of sperm. To further understand the role of serotonin in the gametogenic and spawning processes, we used both immunohistochemistry and high-pressure liquid chromatography linked with electrochemical detection to detect serotonin in the gonads of the surf clam. We found serotonin-containing varicose fibers covering the surface of the germinal epithelium in both sexes. The area occupied by the serotonergic innervation field encircling gonad acini varied according to the gonadal stages (active phase, ripe phase, partially spawned phase, spent phase). We also found large variations in the serotonin concentration between specimens during the gametogenic cycle. The serotonin concentration was correlated with gonad growth: it decreased in the ripe phase in comparison with the previous phase, the active phase. We attribute the decrease to the increase of total gonad mass in this stage. In contrast, as spawning begins, the total gonad mass declines while the gonad serotonin concentration increases to a level similar to that found in active phase. The finding that prior to spawning, serotonin is present in the gonads within fibers exhibiting distinct varicosities suggests that it is implicated in spawning.

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Introduction

Serotonin (5-hydroxytryptamine, 5-HT) has been implicated in the spawning process of bivalve molluscs since the pioneer work of Matsutani and Nomura (1982), who first reported that serotonin induces the release of gametes in the scallop *Patinopecten yessoensis*. Subsequently, serotonin was found to trigger spawning in several other pelecypods such as the bay scallop *Argopecten irradians*, the hard clam *Mercenaria mercenaria*, the ocean quahog *Arctica islandica*, the ribbed mussel *Geukensia demissa* (Gibbons and Castagna, 1984), the zebra mussel *Dreissena polymorpha* (Ram *et al.*, 1992), the doughboy scallop *Chlamys asperrima* (O'Connor and Heasman, 1995), the pismo clam *Tivela stultorum* (Alvarado-Alvarez *et al.*, 1996), the hen clam *Mactra chinensis* (Fong *et al.*, 1996), and particularly the surf clam, *Spisula solidissima* (Gibbons and Castagna, 1984; Hirai *et al.*, 1988). In this latter species (*Spisula solidissima*), serotonin, at high concentration (1–10 μM), also reinitiates the meiotic maturation of isolated prophase 1-arrested oocytes (Hirai *et al.*, 1988), whereas at lower concentration (<500 nM) it enhances the fertilizability of oocytes by sperm (Juneja *et al.*, 1993; Clotteau and Dubé, unpubl. data). This suggests that serotonin might be present in the gonad, where it would play a physiological role during the spawning process. Furthermore, this hypothesis is supported by radioligand binding studies revealing the presence of a new subtype of serotonin receptor on *Spisula* oocytes (Krantic *et al.*, 1991, 1993). In agreement with the possibility that endogenous serotonin is a central neurochemical involved in surf clam reproduction, its presence in the animal's central nervous system (CNS) was reported long ago (Welsh and Moorhead, 1960), and a serotonin-like

substance was detected in its body fluids (Kadam and Koide, 1989). However, to date, no studies have reported the presence of serotonin in surf clam gonads.

In other molluscs, immunohistochemical and biochemical studies report that serotonin is present in the ganglia of *Mytilus edulis* (Welsh and Moorhead, 1960; York and Twarog, 1973; Stefano and Catapane, 1977), *Artica islandica, Venus mercenaria, Ensis directus, Mya arenaria* (Welsh and Moorhead, 1960), and *Mytilus galloprovincialis* (Vitellaro-Zuccarello *et al.*, 1991). In other species, serotonin was detected both in ganglia and gonads of *Patinopecten yessoensis* (Matsutani and Nomura, 1986), *Dreissena polymorpha* (Ram *et al.*, 1992), *Pecten maximus* (Paulet *et al.*, 1993), *Argopecten purpuratus* (Martinez and Rivera, 1994; Martinez *et al.*, 1996), and *Placopecten magellanicus* (Croll *et al.*, 1995; Pani and Croll, 1995). Therefore, it has been suggested that serotonin would be axonally transported from the CNS towards the gonads, where several serotonin-immunoreactive fiberlike projections have been observed surrounding male and female acini (Matsutani and Nomura, 1986; Ram *et al.*, 1992; Paulet *et al.*, 1993; Croll *et al.*, 1995). On the other hand, Ram *et al.* (1993) observed that female responsiveness (spawning) to serotonin varies during the gametogenic cycle of zebra mussels. In those experiments, serotonin failed to induce spawning of ripe females, even though—a few weeks later—those same ripe females (or even less mature ones) might spawn upon injection of serotonin. These results suggest that endogenous serotonin might be modified in the gonad, prior to spawning (Ram *et al.*, 1993). Variations in serotonin localization have been described in the scallop *Placopecten magellanicus*, in which serotonin-immunoreactive fibers were abundant surrounding gonad acini only a few weeks subsequent to spawning, whereas in spring, before spawning, they were only occasionally detected around the germinal acini (Croll *et al.*, 1995). Furthermore, in the ganglia of *Mytilus edulis* and the scallop *Pecten maximus* and in both the ganglia and the gonads of the hermaphrodite *Argopecten purpuratus*, variations of serotonin content correlated with modifications occurring during the gametogenic cycle (York and Twarog, 1973; Stefano and Catapane, 1977; Paulet *et al.*, 1993; Martinez and Rivera, 1994).

The precise involvement of serotonin in the control of spawning in bivalves remains unclear. The surf clam, *Spisula solidissima*, offers a good model for studying how serotonin is implicated in spawning, since the effects of exogenous serotonin on its spawning (Gibbons and Castagna, 1984), oocyte maturation (Hirai *et al.*, 1988), and fertilizability (Juneja *et al.*, 1993) are well documented. In addition, this species has a well-known gametogenic cycle that has been described extensively by Ropes (1968). Finally, this pelecypod is dioecious, allowing both sexes to be studied individually.

The purpose of this study was to determine whether serotonin is present in the gonads of *S. solidissima* and to localize and quantify it through the gametogenic cycle. The distribution of serotonin in surf clam gonads was determined by immunohistochemistry and by high-pressure liquid chromatography with electrochemical detection (HPLC/ED). The results indicate the presence of serotonin within fiberlike projections that surround both male and female germinal acini. These serotonin-containing fibers are present throughout the gametogenic cycle, which—despite some variations in their distribution and in gonad serotonin concentration during this period—indicates that serotonin might be involved extensively throughout the gametogenic cycle of the surf clam.

Materials and Methods

Handling of specimens

Surf clams (*Spisula solidissima*, 80–100 mm) were collected at Iles-de-la-Madeleine (Québec, Canada) from April to September and shipped to the Centre Hospitalier de l'Université de Montréal (Montréal, Canada) where they were kept in an aquarium at 8 °C. Surf clams were used within 2 days for both immunolocalization and quantification of serotonin assessed in gonads from 69 collected animals.

Immunohistochemistry

Pieces of gonad were excised and fixed overnight at 4 °C in a solution of 4% paraformaldehyde in 0.1 *M* phosphate buffer (pH 7.4) containing 2.4% NaCl. They were washed thrice, 15 min each, in phosphate buffered saline (PBS), and transferred to a solution of 15% sucrose in PBS for 20 min in preparation for subsequent freezing. They were then immersed in PBS containing 30% sucrose until gonadal tissues settled on the bottom of the dish. Specimens were embedded with O.C.T. compound (Miles), frozen in chilled 2-methyl butane, and stored at −80 °C until used. Cryostat sections, about 30 μm in thickness, were placed alternately on different glass slides coated with 1 mg/ml L-polylysine (Sigma) for dual procedures of immunohistochemistry and histology.

After they were left to dry for 2 h, tissue sections were given three 15-min washes in PBS at room temperature. They were incubated in 1% normal goat serum (Jackson ImmunoResearch Laboratory) containing 0.3% Triton X-100 in PBS for 20 min. This was followed by incubation with a rabbit anti-serotonin antibody (Incstar, diluted 1:1750) and 0.2% Triton X-100 in PBS for 20–24 h at 4 °C. After three rinses, of 15 min each, in PBS, the tissue sections were incubated with Cy3-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch, diluted 1:100 in PBS) for 1 h at room temperature. All subsequent proce-

dures were performed at room temperature. After another rinse in PBS, sections were incubated, for 1 h, with 1 μg/ml Hoechst 33258 (Sigma) for DNA staining. They were again washed three times, 10 min each, in PBS. Sections were mounted with Aquamount. Preparations were viewed and photographed using a Leitz Dialux EB epifluorescence microscope with two filter sets: for 5-HT detection, an excitation filter of 515–560 nm and an emission filter of 580 nm; for Hoechst detection, an excitation filter of 340–380 nm and an emission filter of 430 nm. The specificity of the anti-serotonin antibody was assessed by pre-absorbing 1 ml of diluted antibody with 200 μg of serotonin/bovine serum albumin conjugate (Inestar) over 16–24 h with gentle agitation at 4 °C.

To determine the gonadal stages of surf clams, frozen tissue sections on gelatin-coated slides were processed for standard hematoxylin and eosin staining. Sections were then viewed using a Zeiss Axiomat microscope, and the four gonadal stages as defined by Ropes (1968)—active phase, ripe phase, partially spawned phase, and spent phase—were determined (for more details see Results section). Light photomicrographs (shown in Fig. 1) were taken from standard hematoxylin-and-eosin-stained paraffin gonad sections of 10 μm in thickness that came from the entire visceral mass preserved in Bouin's fixative.

Measurement of serotonin content

After small portions of gonadal tissues were collected for immunohistochemistry, the serotonin content of the remaining gonad was measured by HPLC. Some gonads were cut in half prior to further processing. After dissection, gonads or gonad halves were individually put in preweighed Eppendorf tubes containing 0.6–1.0 ml of ice-cold 0.1 *N* perchloric acid, weighed again, and homogenized with a Kinematica polytron. The homogenate was centrifuged at 16,000 × *g* for 15 min at 4 °C, and the supernatant was stored at −80 °C until processed. As a measure of serotonin recovery, a known amount of serotonin creatinine sulfate (Sigma) was added to half-gonad specimens, and the results were compared with the values for the matching halves that received no exogenous serotonin.

The HPLC system consisted of a Waters pump (model 590), equipped with a Waters automatic injection system (model 710B). A Waters M460 electrochemical detector with a potential of +0.85V applied across the electrode was used for amine detection. Chromatographic separations were carried out on a Supelcosil LC-18-DB reverse-phase column (Supelco, 150 × 4.6 mm i.d.) packed with 5-μm particles and equipped with a guard column (Supelguard LC-18-DB, 20 × 4.6 mm i.d.). Both columns were maintained at 40 °C while the rest of the system was run at ambient temperature (22 °C).

The mobile phase consisted of 300 m*M* phosphate buffer containing 4 m*M* 1-heptane sulfonic acid (Sigma), 0.2 g/l Na_2 EDTA (Fisher), and 5% acetonitrile (Baxter, Burdick and Jackson). The pH was adjusted to 2.5 with 85% *o*-phosphoric acid (Fisher). This solution was prepared with distilled and deionized water and purified by a Norganic cartridge (Millipore) to remove any organic substances. The mobile phase was filtered through Millipore membrane filters of 0.45-μm porosity under vacuum. The mobile phase was degassed with helium and pumped at a rate of 1.3 ml/min, producing a background pressure of about 1000 psi. All reagent chemicals used were of HPLC grade.

The stock solutions of serotonin, 5-hydroxytryptophan, dopamine, and of the internal standard 3,4-dihydroxybenzylamine (DHBA), all from Fisher Scientific, were prepared in 0.1 *N* perchloric acid at a concentration of 1 m*M* and stored at 4 °C. The working standard solutions were prepared by diluting the stock solutions in 0.1 *N* perchloric acid. The standard retention times were 3.3 min for DHBA and 10.3 min for serotonin.

For each run, three serotonin and DHBA standards were processed to identify their retention times and confirm the serotonin elution peak from samples. This was followed by extracts containing the same amount of the DHBA standard. In all cases, 25 μl of samples and standards were injected in the column. The signal from the electrochemical detector was fed directly to a Waters data module 740 acquisition system. The serotonin content was determined by comparing serotonin/DHBA peak height ratios of unknown sample chromatograms with those of chromatograms from serotonin standards.

Statistical procedures

The data (nanograms of serotonin per gram of gonad wet weight) of combined male and female surf clams were tested for normality ($P < 0.01$) and heteroscedasticity ($P < 0.001$). Since data were not normally distributed, and because of the small sample size, a nonparametric test was used. No such test exists for two-way analysis of variance (ANOVA). Therefore, all data were rank-transformed before computing a two-way ANOVA (Shirley, 1987). Pairwise comparisons were done using the Student-Newman-Keuls test. The accepted level of statistical significance was $P < 0.05$.

Next, female and male gonad serotonin concentrations (ng/g of gonad wet weight) were analyzed separately. As for the combined data, they were both tested for normality (female: $P > 0.05$; male: $P < 0.01$) and heteroscedasticity (female: $P < 0.001$ male: $P > 0.05$). Since data from each sex were not normally distributed and comparison with combined data was necessary, all data were rank-transformed, and a one-way ANOVA was performed for each sex. Pairwise comparisons were done using the Student-

Newman-Keuls test. The accepted level of statistical significance was $P < 0.05$.

The same statistical procedures were used for the analysis of gonad wet weight (g) of combined female and male surf clams. A two-way ANOVA was performed on rank-transformed data since they were not normally distributed (normality: $P > 0.01$ and heteroscedasticity: $P < 0.001$) and because of the small sample size. Subsequently, a one-way ANOVA was done on rank-transformed data from each sex separately since they were not normally distributed (normality: female, $P < 0.01$; male, $P > 0.2$; heteroscedasticity: female, $P < 0.01$; male, $P < 0.01$). Pairwise comparisons were performed as above.

Results

Surf clams used for immunohistochemistry and HPLC were first examined to determine their gonadal stages. As shown in Figure 1, we used specimens of every stage of the gametogenic cycle. Males in active phase (stage 1) of their gametogenic cycle are easily distinguishable by the granular aspect of their acini (Fig. 1A). In this phase, spermatogonia proliferate from alveolar walls, and early spermatocytes begin to occupy the lumen of acini, forming radial columns. In the ripe phase (stage 2), acini exhibit, instead of a granular aspect, a braided shape as a result of sperm tails orienting toward the lumen of acini (Fig. 1B). When a lumen appears in the center of acini with some sperm tails still visible, spawning has begun and specimens are classified as partially spawned males (stage 3, Fig. 1C). At the end of the gametogenic cycle, when males have released all of their gametes, acini are almost devoid of mature cells, with some spermatogonia proliferating in alveolar walls (stage 4, Fig. 1D). The female gametogenic cycle follows almost the same pattern as the male. In females in active phase (stage 1), oogonia and early oocytes appear in the periphery of acini, the latter being attached to the wall by a stalk (Fig. 1E). Afterwards, ripe females (stage 2) are easily recognizable by their large mature oocytes filling the lumen space of acini and by their thin alveolar wall (Fig. 1F). When spawning has begun, less mature oocytes are observed in the lumen of acini (stage 3, Fig. 1G). Finally, females in the last phase (stage 4) of their gametogenic cycle have already released almost all ripe oocytes, and oogenesis has already begun in the alveolar walls (Fig 1H).

Serotonin content

The specimens identified by their gametogenic stage were used to measure gonad serotonin content by HPLC/ED. This analysis revealed the presence of a compound co-eluting with serotonin. The chromatogram in Figure 2 illustrates the serotonin elution peak for both the standard and a typical sample. From around 10.3 min of elution time onward, the serotonin elution peak is the only one present in all gonad extracts tested. We also found traces of compounds co-eluting with dopamine and 5-hydroxytryptophan in most of the gonad extracts (not shown). Small elution peaks of unknown origin were common in the extract chromatograms.

Serotonin was detected in all gonad extracts tested. We found large variations in gonad serotonin content among specimens, from 7.5 to 2117 ng/g of gonad (wet weight). To determine if these fluctuations were related to sex or gonadal stage, we used a two-way ANOVA on data transformed on ranks. We found that these variations of gonad serotonin concentration (ng/g) are observed in both male and female gonads and that for each gonadal stage serotonin concentrations were not significantly different between males and females. Since no difference between sexes was found, we tested the hypothesis that the variations in serotonin concentration might be related to the gametogenic stage of the gonad. Analysis of data from combined sexes revealed a significant difference ($P < 0.002$) of serotonin concentration (ng/g) between the different gonadal stages. In fact, all pairwise multiple comparisons (Student-Newman-Keuls Method) indicate that the serotonin concentration in ripe phase (stage 2) differs greatly from the concentrations in the active (stage 1, $P < 0.01$) and spent phases (stage 4, $P < 0.01$, Fig. 3). A high gonad serotonin concentration is seen in active phase (stage 1), followed by an abrupt decline in ripe phase (stage 2), just before spawning. The gonad serotonin concentration seems to increase again after spawning (stage 3), but this increase is not statistically significant (Fig. 3). A significantly higher gonad serotonin concentration is reached only when gametes have all been expelled from clams in the spent phase (stage 4, Fig. 3). Next, female and male clams were considered separately using one-way ANOVA with rank-transformed data to test whether the relationship between the gonadal stage and the serotonin concentration was similar in both sexes. For female clams, a significant difference in serotonin concentrations ($P < 0.002$) between the gonadal stages (Fig. 4) mirrors observations with sexes combined (Fig. 3). In contrast, no significant difference emerged from analysis of male gonad serotonin concentrations (Fig. 4).

Another two-way ANOVA was performed to determine if the significant relation between gonadal stage and serotonin concentration was associated with variations of gonad weight during the gametogenic cycle. When the average total gonad weights were analyzed, no significant differences were noted between sexes, regardless of whether all specimens were combined or those from a specific gonadal stage were considered. In contrast, a significant difference ($P < 0.001$) was noted when the total gonad weights, both sexes combined, were compared for each gonadal stage (Fig. 5). All pairwise multiple comparisons (Student-Newman-Keuls Method) indicate that gonad weight increases significantly as the clams ripen between the active phase

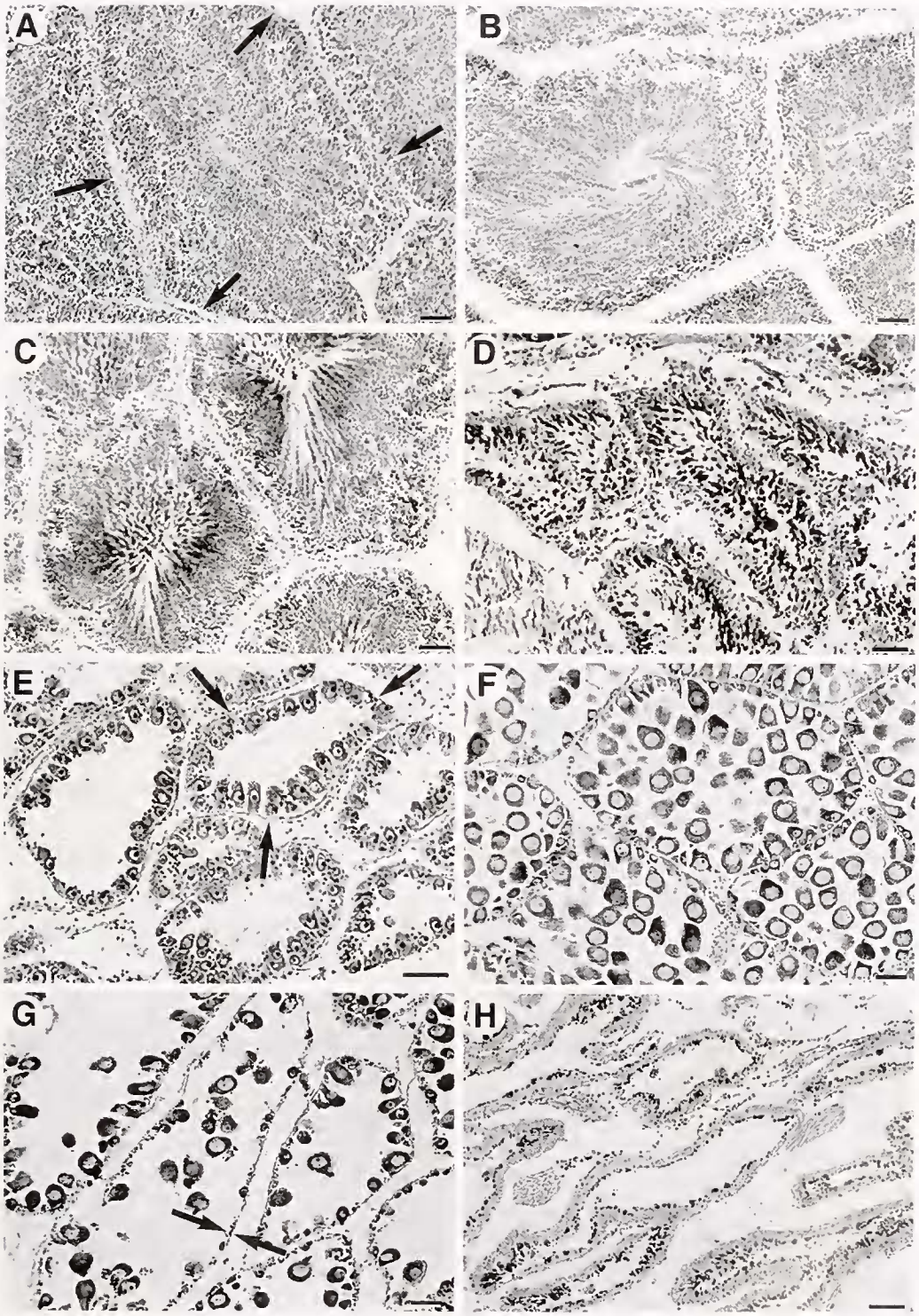

Figure 1. Sections of gonads at the different stages of surf clam gametogenic cycle: A-D, males; E-H, females. The various stages depicted are active phase (stage 1; A, E), ripe phase (stage 2; B, F), partially spawned phase (stage 3; C, G), and spent phase (stage 4; D, H). Note typical male (A) and female (E) acini that are surrounded by arrows. Also, note the thin alveolar wall (*between arrows*) in female partially spawned phase (G). Scale bars: 100 μm.

(stage 1) and the ripe phase (stage 2, $P < 0.001$, Fig. 5). This is followed by an apparent, but statistically insignificant, decrease as the clams begin to spawn (stage 3, Fig. 5). When the clams have released all their gametes, a significant decrease is noted between the ripe phase (stage 2) or the partially spawned phase (stage 3) and the spent phase

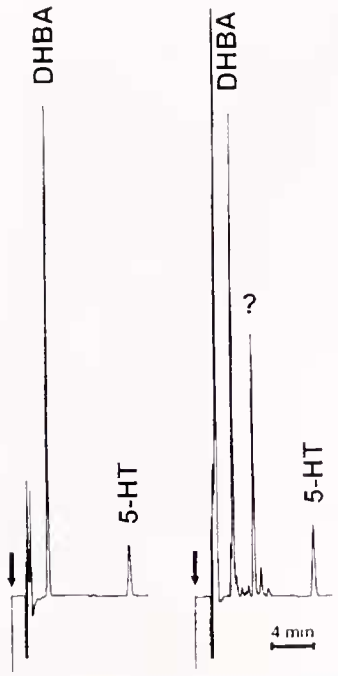

Figure 2. HPLC-ED detection of serotonin in surf clam tissue extracts. Left trace: Detection of standards following injection (*arrow*) of 4.4 ng of serotonin (5-HT) and 3.5 ng of 3,4-dihydroxybenzylamine (DHBA). Right trace: Chromatogram of surf clam gonad extract run consecutively to the standard. DHBA was used as an internal standard (see Materials and Methods). Note in the right trace that an unidentified peak (?) is shown that does not correspond to any other serotonin-related or catecholaminergic compounds tested, *e.g.*, 5-hydroxy-3-indoleacetic acid, *N*-acetyl-serotonin, epinephrine, norepinephrine.

(stage 4, $P < 0.001$, Fig. 5). Using one-way ANOVA on ranked data, we found the same relation between gonad weight and gonadal stage in males and females analyzed separately (not shown).

Localization of serotonin immunoreactivity

In agreement with the detection of serotonin in gonads by HPLC/ED, serotonin-immunoreactive (5-HT-IR) fibers were detected in surf clam gonads of both sexes. Control sections (treated as described in Materials and Methods) were devoid of any staining in gonads (not shown). 5-HT-IR fibers were observed in specimens from all gametogenic stages. Transverse sections of acini show 5-HT-IR fibers located at the periphery of each acinus, with variations in their distribution among the gametogenic stages as shown for females (Figs. 6 and 7). Thus, in active-phase females (Fig. 6A), 5-HT-IR fibers surrounded each acinus, where they formed a thin discontinuous innervation field. At low magnification, little difference in 5-HT-IR staining was apparent between active-phase and ripe-phase females (Fig. 6A, B), even though the alveolar wall was much thicker in the former (compare Fig. 7A and B). However, in ripe

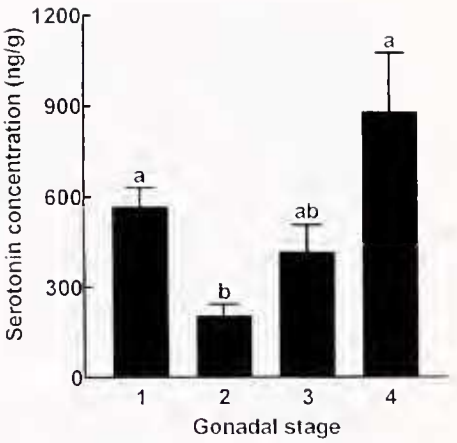

Figure 3. Average serotonin concentration in surf clam gonads during the gametogenic cycle. Data from both sexes are combined. The gametogenic cycle was divided into four stages: (1) active phase, (2) ripe phase, (3) partially spawned phase, and (4) spent phase. The letters a and b indicate relationships among the stages. The two stages (1 and 4) marked "a" are not different from each other, but they are significantly different from the stage (2) marked "b". The stage (3) marked "ab" is not significantly different from any of the three other stages. Vertical error bars correspond to the standard error of the means (SEM).

females, the staining around acini was more frequently interrupted (Fig. 6B), and it was concentrated in varicosities within 5-HT-IR fibers (Fig. 7B). It was also noted that staining was observed neither on or in oocytes present in gonad sections (Fig. 7B) nor in isolated oocytes (not shown). In the subsequent stage, when spawning had begun and acini contained fewer ripe oocytes in their lumen, different patterns of 5-HT-IR fibers could be seen (Figs. 6C,

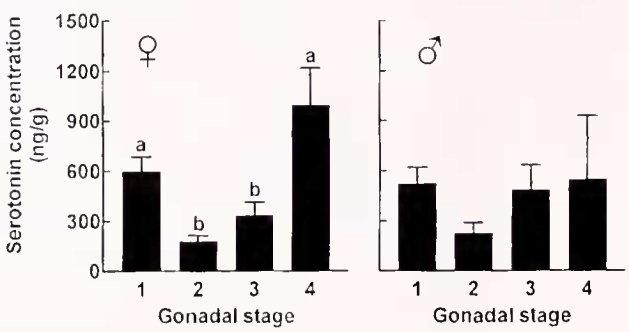

Figure 4. Average serotonin concentration in surf clam gonads during female and male gametogenic cycle. The gametogenic cycle was divided into four stages: (1) active phase, (2) ripe phase, (3) partially spawned phase, and (4) spent phase, for both females (♀) and males (♂). The only significant differences were found in females between gonadal stages 1 and 2, 1 and 3, 2 and 4, and 3 and 4. The letters a and b indicate relationships among the stages. The two stages marked "a" are not different from each other, but they are significantly different from the other two stages marked "b". In males, none of the stages were significantly different one from another. Vertical error bars correspond to the standard error of the means (SEM).

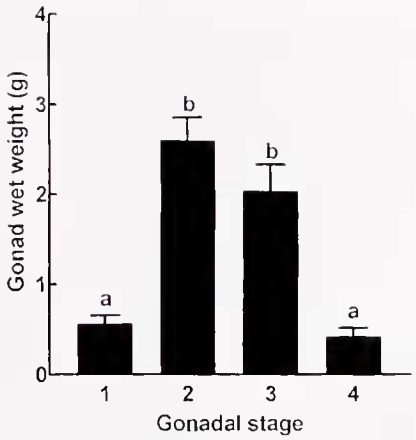

Figure 5. Average gonad wet weight in surf clam gonads during the gametogenic cycle. Data from both sexes are combined. The gametogenic cycle was divided into four stages: (1) active phase, (2) ripe phase, (3) partially spawned phase, and (4) spent phase. The letters a and b indicate relationships among the stages. The two stages (1 and 4) marked "a" are not different from each other, but they are significantly different from the other two stages (2 and 3) marked "b". Vertical error bars correspond to the standard error of the means (SEM).

7C). Staining depended on the spawning condition of the acinus. Acini that were still full of oocytes showed the same immunoreactive staining as ripe females (Fig. 6C), whereas acini that had begun to expel some oocytes showed an uninterrupted staining all around the acinus (Fig. 7C). Also, in contrast to ripe-stage gonads exhibiting generally a single 5-HT-IR fiber running parallel to the acinus (Fig. 7B), acini devoid of most of their oocytes in partially spawned gonads had more numerous fibers in diverse orientations (Fig. 7C). As the spent phase was reached, almost all mature oocytes have been expelled, and gonadal tissues began to disrupt. At this stage, some acini remained recognizable, whereas others were completely disrupted or apparently shrunken (Fig. 6D). In nondisrupted acini, as well as in disrupted acini constituting the major part of the gonad, the meshwork of 5-HT-IR fibers appeared more disorganized and spread over a larger area than in other stages (Fig. 6D). In addition, these fibers displayed no specific orientation plan, sometimes being parallel to the alveolar wall and sometimes perpendicular or at any other angle (Fig. 7D).

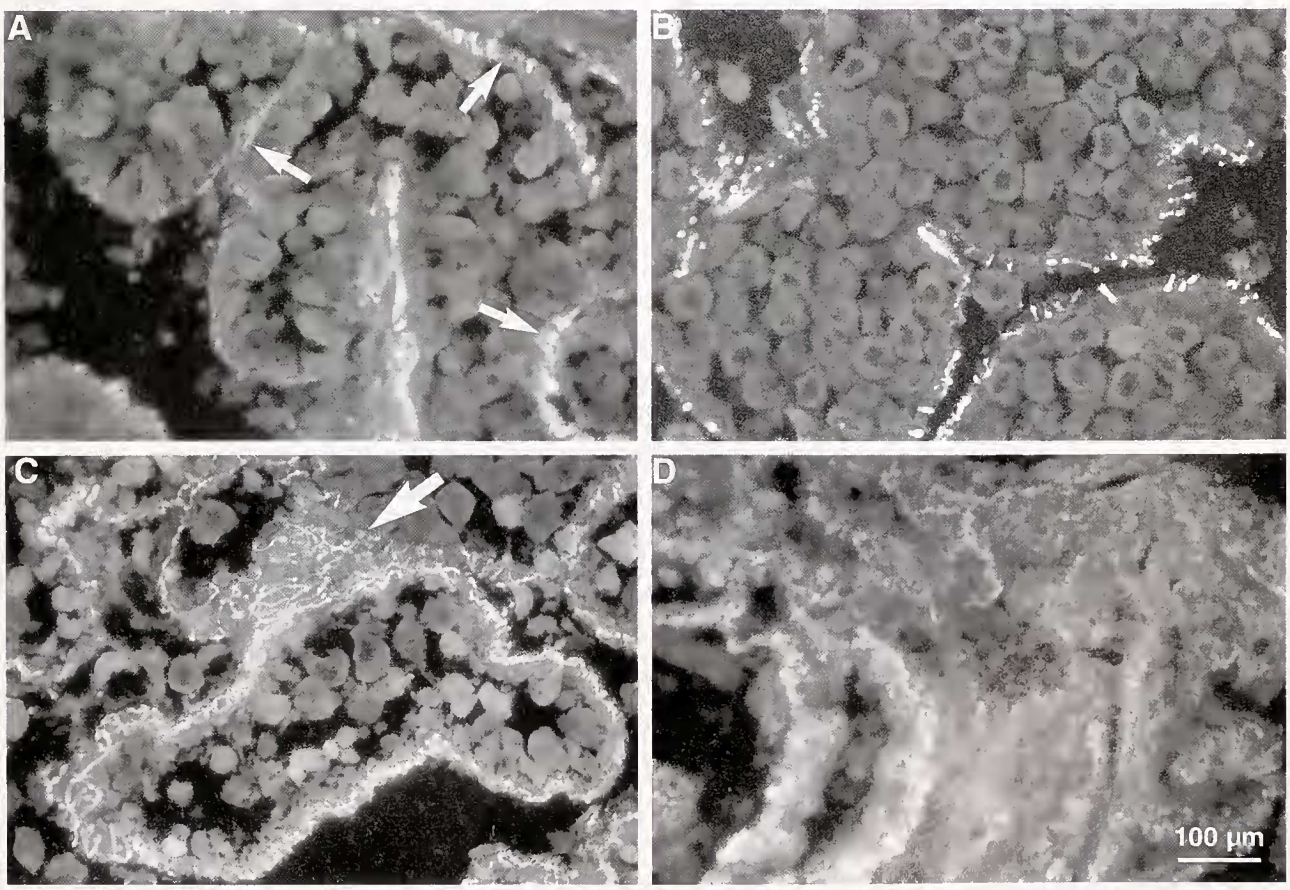

Figure 6. Localization of serotonin immunoreactivity in surf clam female gonads during the gametogenic cycle. The gametogenic cycle was divided into four stages: (A) active phase, (B) ripe phase, (C) partially spawned phase, and (D) spent phase. Note in A the localization of staining indicated by arrows surrounding active-phase acini. Note in C a meshwork of serotonin-immunoreactive fibers in a tangential gonad section (*arrow*).

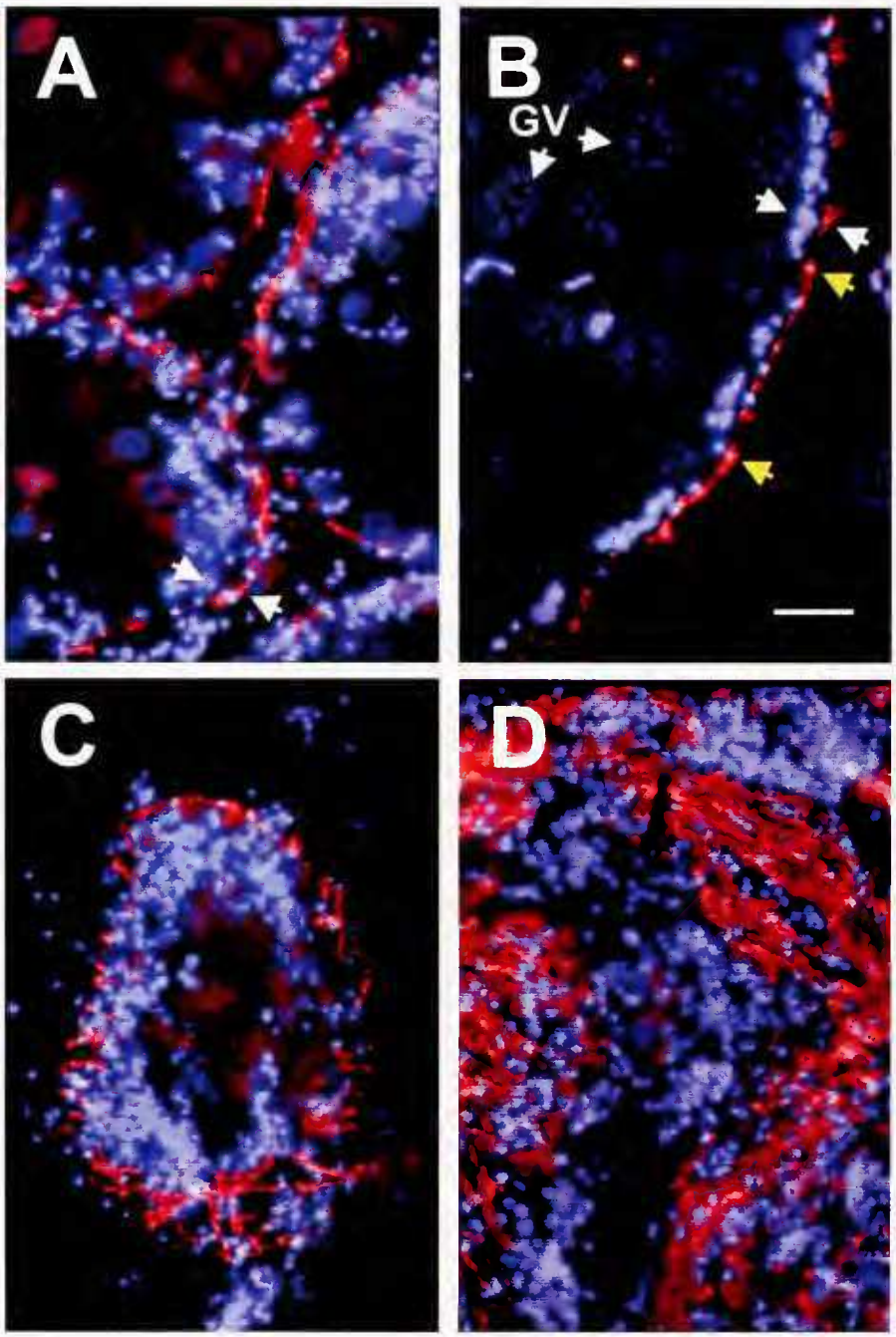

Figure 7. Epifluorescence micrographs of serotonin immunoreactivity and DNA staining (Hoechst 33258) in surf clam female gonads during the gametogenic cycle. Serotonin-immunoreactive elements are stained in red and DNA in blue. The gametogenic cycle was divided into four stages: (A) active phase, (B) ripe phase, (C) partially spawned phase, and (D) spent phase. Note in A and B the width of the alveolar wall (*between white arrows*). Also, note in B the two varicosities of serotonin-immunoreactive projection (*yellow arrows*). GV: germinal vesicle of ripe oocytes. Scale bar, 50 μm.

During the male gametogenic cycle (Fig. 8), the distribution of 5-HT-IR fibers was similar to that of females. In active-phase males (Fig. 8A), granular acini were surrounded by a few 5-HT-IR fibers that formed a thin innervation field. As they reached the ripe phase (Fig. 8B), male acini displayed an interrupted staining pattern resulting mostly from varicosities within one 5-HT-IR fiber. In males that had already begun to spawn (Fig. 8C), several 5-HT-IR fibers occupied a larger innervation field around the acinus than in ripe-phase gonads. After sperm had been completely

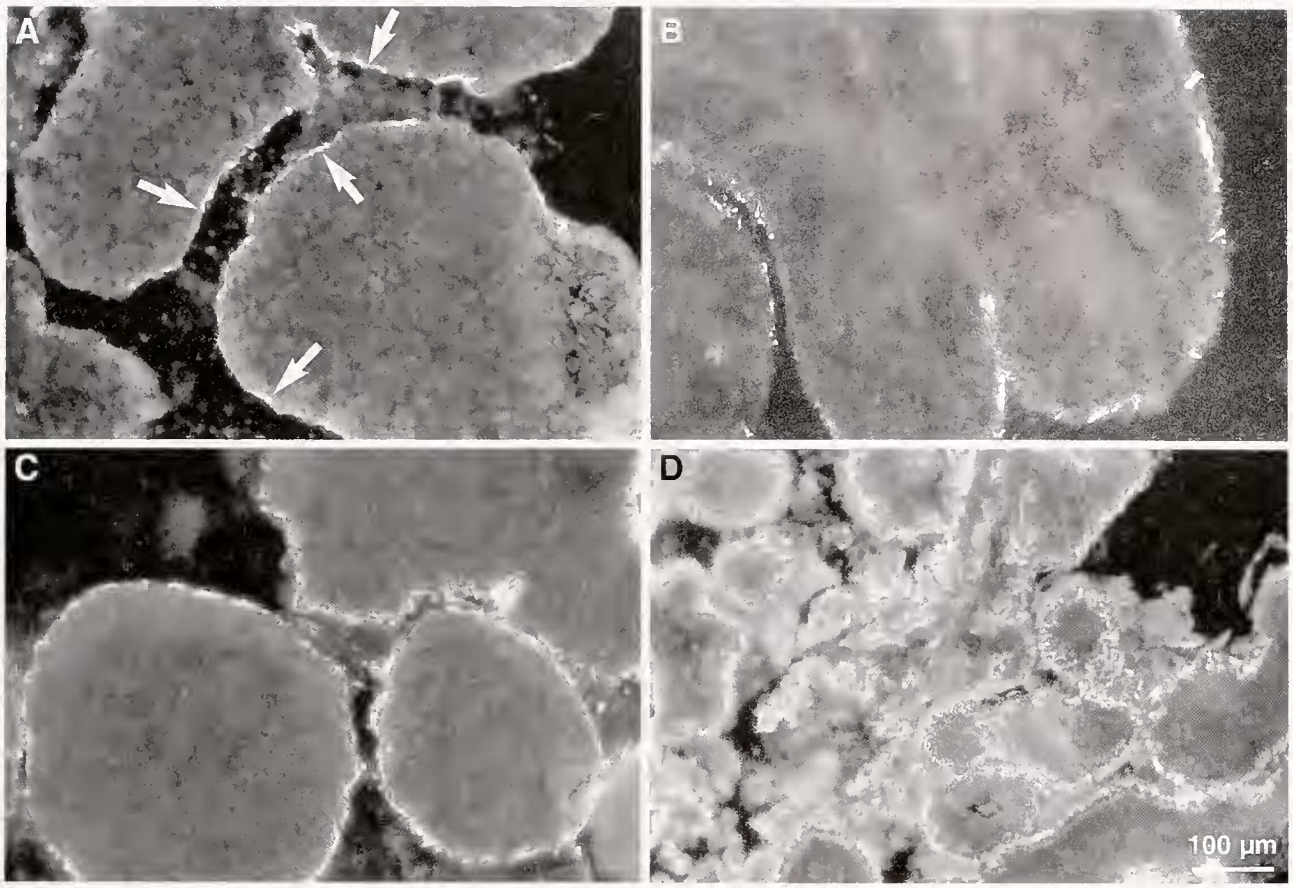

Figure 8. Localization of serotonin immunoreactivity in surf clam male gonads during the gametogenic cycle. The gametogenic cycle was divided into four stages: (A) active phase, (B) ripe phase, (C) partially spawned phase, and (D) spent phase. Note in A the localization of reactive fibers surrounding two acini (*arrows*).

expelled from acini (Fig. 8D), the immunoreactive field around recognizable acini was expanded as in females.

Discussion

The present study provides immunohistochemical and biochemical evidence for the presence of serotonin within the gonad of the surf clam, *Spisula solidissima*. Although serotonin has been reported from the gonads of some other bivalves, our work is the first to report changes in the pattern of gonadal serotonin innervation during the various stages of the gametogenic cycle. During the active phase (stage 1) when clam gonads contain small but recognizable gametes, some serotonin-containing fibers are detected covering the surface of the germinal acini, an observation also reported for scallops at a similar stage (Croll *et al.*, 1995). However, later in the season, in ripe gonads (stage 2), these varicose fibers conserve a similar shape and can be easily detected, but they appear less numerous along the germinal acini than in the previous phase, an observation never reported for other bivalves. Concurrently, the gonadal serotonin concentration (measured as nanograms of serotonin per gram of gonadal tissue) is, at this phase (ripe phase), significantly lower than in the active phase. This was also observed for the female gonad portion of the hermaphrodite *Argopecten purpuratus*, in which the serotonin concentration decreases during the 2 weeks before the second annual spawning (Martinez and Rivera, 1994). When spawning has begun, and the total mass of gonads starts decreasing, a more extensive network of conspicuous serotonin-containing fibers is detected around the germinal epithelium. Croll *et al.* (1995) made similar qualitative observations with scallops, that were not, however, supported by any parallel significant changes in serotonin levels. When surf clams have completed spawning (stage four, spent phase) and almost all mature gametes have been expelled, leaving nearly empty, shrunken, and lower-mass gonad, numerous serotonin-containing fibers encircle the acini, as also reported for the scallop *Placopecten magellanicus* of the same gonadal stage (Croll *et al.*, 1995). This observation is supported by a significantly higher serotonin concentration in comparison with values obtained from ripe clams (stage 2). Thus, unlike *Argopecten purpuratus* (Martinez and Rivera, 1994), in

which the serotonin concentration decreases after spawning, clams show an increase in serotonin, and the concentration presumably remains high until the subsequent ripe stage.

In contrast, even though similar qualitative changes could be observed in the serotonergic innervation in male gonads during the gametogenic cycle, these observations are not paralleled by significant changes in serotonin concentrations between the various gametogenic stages. This may be partly due to the great variations in serotonin concentrations between individual specimens and the small sample size for the fourth phase (spent phase) of the gametogenic cycle. Indeed, since overall immunohistochemical observations are similar in male and female surf clams and the male gonad mass varies in the same manner as the female, we would expect a similar regulation of the gonadal serotonergic network in both sexes. Indeed, the gonadal serotonin concentrations are largely at comparable levels in both male and female surf clams and their responsiveness to injected serotonin is similar, thus suggesting that gonadal serotonin has equivalent roles in the reproductive processes in both sexes.

Overall, we find that gonadal serotonin concentration is correlated with total gonad mass. Indeed, the significant decrease in serotonin concentration during the ripe phase (Fig. 3) most probably reflects the major increase in the total mass of gonads when they are ripe (Fig. 5). Since this increase in total gonadal mass is mostly due to multiplication and growth of gametes, which are devoid of serotonin, this results in a lower serotonin concentration when expressed in nanograms per gram of gonadal tissue. In contrast, when spawning is complete and the total gonad mass is low, the serotonin concentration is high. Therefore, the total serotonin content within gonads appears to remain rather stable at substantial levels throughout the gametogenic cycle. In *Spisula,* since endogenous serotonin is present in the ripe-phase gonad, and since exogenous serotonin induces the release of gametes, serotonin is believed to be directly involved in the spawning process. Our observations of a stable gonadal serotonin content throughout gametogenesis suggest that the gonadal serotonin release that presumably induces spawning might be restricted in time, with normal levels being rapidly reestablished. In addition to inducing spawning, this release of serotonin in the gonads could also enhance the fertilizability of oocyte by sperm *in vivo,* as reported *in vitro* for isolated *Spisula* oocytes (Juneja *et al.,* 1993). In conclusion, our present work clarifies the localization and pattern of serotonin and its quantitative fluctuations during the gametogenic cycle. This opens new avenues for further investigations on issues such as the neuronal regulation of serotonin release and the nature of the gonadal serotonin receptors involved in the reproductive process.

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